

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 41/00	A1	(11) International Publication Number: WO 99/39738 (43) International Publication Date: 12 August 1999 (12.08.99)
<p>(21) International Application Number: PCT/IB99/00182</p> <p>(22) International Filing Date: 2 February 1999 (02.02.99)</p> <p>(30) Priority Data: 98810095.4 9 February 1998 (09.02.98) EP</p> <p>(71) Applicant: BRACCO RESEARCH S.A. [CH/CH]; 31, route de la Galaise, CH-1228 Plan-les-Ouates (CH).</p> <p>(72) Inventors: SCHNEIDER, Michel; 34, route d'Annecy, CH-1256 Troinex (CH). YAN, Feng; 12, route des Acacias, CH-1227 Carouge (CH). HIVER, Agnès; Chef Lieu Arcine, F-74270 Clarafond (FR).</p>	<p>(81) Designated States: AU, CA, JP, NZ, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: TARGETED DELIVERY OF BIOLOGICALLY ACTIVE MEDIA</p> <p>(57) Abstract</p> <p>The invention concerns a method for administering bioactive substances to patients at selected sites in the body and remotely promoting delivery of said media to selected organs or tissues in the body. The method comprises providing an administrable formulation comprising, dispersed in an aqueous carrier liquid, liposomes filled with bioactive substances and gas-filled microspheres, injecting said formulation into the circulation of a patient so that it is directed to a site of interest, and applying ultrasound pulses to said site so as to make the gas-filled microbodies explode and the gas confined therein to expand in the carrier liquid, the energy of expansion of said confined gas causing the liposome vesicles to open and release the trapped substances at said site. Also disclosed are formulations for delivery of biologically active substances to selected target sites in the organism, the formulations comprising an aqueous suspension of gas-filled microvesicles and liposomes filled with active substances such as drugs or diagnostic agents. The formulations are available in a kit form in which the kit comprises sterile precursor components.</p> <p style="text-align: right; font-size: 1.5em; transform: rotate(-10deg);">BEST AVAILABLE COPY</p>		

AD

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

TARGETED DELIVERY OF BIOLOGICALLY ACTIVE MEDIA

Field of the invention

5

The present invention concerns a method and compositions or formulations for administering and controllably delivering bioactive substances or media to selected sites, e.g. organs or tissues, in the body of patients. The formulations comprise ingestible or injectable aqueous suspension of liposomes bearing active substances such as drugs or diagnostic agents encapsulated therein. The formulation is also available in kit form, the kits comprising sterile precursor components.

15

Background Art

The targeted delivery via the circulation of liposomes encapsulating bioactive media like therapeutic or diagnostic substances towards selected areas in the organism combined with the assisted release of said substances at specific sites is attracting much attention in the medical field. For instance, N. Shoucheng et al., *Int. J. Radiat. Oncol. Biol. Phys.* 29 (1994), 827-834 have disclosed injecting long lived liposomes (stealth) containing doxorubicine into the circulation of experimental animals and thereafter inducing controlled release of the doxorubicine at selected sites in the body via local hyperthermia induced by focused ultrasonic energy. Similarly, Bednarski et al. *Radiology* 204 (1997), 263-268 have disclosed the magnetic resonance guided targeting of liposome vesicles incorporating pharmaceuticals towards specific areas in the body, this being followed by the ultrasound controlled release into tissues of said pharmaceuticals, the effect being due to hyperthermialysis of the liposomal membrane.

In WO94/28873 and WO96/39079, there is disclosed a technique in which injectable targeted gas-filled microspheres, for instance gas-filled liposomes, comprising therapeutics embedded within the liposome bilayer membrane wall are directed to specific organs where they are caused to explode by ultrasonic irradiation in order to release said embedded therapeutic substances. It is difficult to incorporate drug into the gas filled liposomes (i.e. in the gas-phase or the surface membrane) without affecting their stability. If even a drug can be load in this kind of vesicles, it must be of a hydrophobic nature and the payload should be very low. Thus this method shows very limited practical utility. And also because after explosion, the therapeutic substance may stick some time to the constituents of the broken liposome membrane in which they were embedded, or the splintered parts of the liposome membranes may be simply "washed away" by the blood stream so that the active substance may not be released on the targeted site but elsewhere.

WO93/25241 discloses an ultrasound imaging technique in which a suspension of microspheres is targeted to organs of the body and caused to collapse under stimulation by ultrasonic energy, whereby a broad-band acoustic signal pulse is emitted and echo-detected by colour Doppler systems.

Although the techniques of the art have merit, a problem may arise due to the level of energy required to break the membrane of the liposomes and release the content thereof to a targeted area; if the area is located deep down in the body, the penetration of the energy beam into the body can have damaging effects to the intervening tissues. Hence searches have been undertaken to find a non-invasive energy releasing agent, closely associated with the liposome vesicles, which can innocuously help breaking the liposome membrane and release the trapped content thereof. In other words, it is strongly desired to

make available an agent containing sufficient potential energy stored therein to open the liposome vesicles without harming the nearby or intervening tissues, said energy being liberated at will by external triggering means, so that the liposome encapsulated bioactive media be set free at a chosen site. The effect sought can be compared to that of a hypothetical prearmed spring to be remotely triggered and whose energy when released will cause the liposome content to be discharged at will. The present invention is set out to achieve this desired effect.

Summary of the invention

In brief, the method of the invention involves directing drug containing liposomes to selected areas in the organism and subsequently breaking or opening the liposomes to release the encapsulated content at a given site. In this method, the potential energy-containing agent to be used in association with the liposome vesicles and whose energy can be liberated at will to assist releasing the liposome encapsulated content consists of microparticles (microbodies) with confined air or gas. The microparticles are preferably air- or gas-filled microspheres, micro-vesicles, or microcapsules, more preferably air- or gas-filled microbubbles or microballoons. When air or gas-filled microspheres in close vicinity to liposome vesicles are caused to break or explode, the liberated cavitation energy will spread around and assist in opening the liposome membrane to free the encapsulated content or by changing the membrane permeability to enhance the drug diffusion. The triggering pulses of, for instance, radio or sound energy to burst the microspheres or microcapsules filled with the confined gas need not be as energetic as those required for

directly acting on the liposomes membrane, hence the impact on nearby tissues is reduced.

The method of the invention is implemented via injectable compositions or formulations comprising liposomes (optionally targeted towards specific sites or organs) carrying encapsulated therein therapeutically or diagnostically useful agents and air or gas filled microspheres, i.e. microbubbles or microballoons which, optionally, may be associated with the liposomes. The microbubbles or microballoons are those disclosed in EP-A-0 474 833; EP-A-0 458 745; EP-A-0 502 814; EP-A-0 554 213; EP-A-0 619 743 and EP-A-0 682 530, all incorporated herein by reference.

The invention also includes precursor systems or kits which may include suspensions of liposomes encapsulating bioactive substances and suspensions of air- or gas-containing microspheres (stable microbubbles or microballoons), or dried liposomes having bioactive substances encapsulated therein in stabilised powder form, as well as suspensions in a carrier liquid of air- or gas-containing stable microbubbles or microballoons, or dried liposomes having bioactive substances encapsulated therein and microballoons in dry powder form, or microbubble precursors as pulverulent laminarized phospholipids stored in contact with air or a physiologically acceptable gas.

Detailed description of the invention

The main aspects of the invention as set out in the accompanying claims are based on an unexpected finding that extremely efficient targeted delivery of biologically active ingredients may be achieved via a method in which an injectable composition comprising (a) liposomes containing encapsulated therapeutically or diagnostically useful agents and (b) air or gas filled microspheres, i.e. microbubbles or microballoons is administered to a

patient. The injected formulation is allowed to reach via the circulation a selected/desired organ or tissue and then the targeted organ or tissues is irradiated with an energy beam (preferably ultrasonic) to burst or cause burst of the gas or air-filled microspheres, the released gas energy thereby opening the adjacent liposomes vesicles, thus causing dispense of the encapsulated biologically active substance(s) at the desired site in the organism of the patient.

Upon administration of an effective amount of such formulation into the vascular or the lymphatic systems of said patient, the progression in the circulation of the administered formulation toward the selected site may be monitored by ultrasonic or MRI imaging means, so that the irradiation and consecutive burst of the gas filled microspheres by sonolysis or otherwise is effected only when the formulation reaches or passes over or through the desired site. Clearly, the process of irradiation may be carried out continuously or intermittently during each cyclic circulation of the formulation through or by the targeted site.

The ultrasonic irradiation may be carried out by a modified echography probe adapted to simultaneously monitor the reflected echo signal and thereby provide an image of the irradiated site. This may further improve efficacy of the method.

Obviously, the total amount of energy discharged at the organ site may not need to exceed that required to break the gas-filled microspheres for and release the bioactive substance, thus minimizing irradiation of the tissue at the targeted organ or site. The frequency of the ultrasonic irradiation required to break the microspheres may vary from about 0.3 to 3 MHz. It should be noted that although any blood or lymph perfused tissue may be targeted according to the invention, it is believed that the most efficiently treated affections relate to

endothelial lesions, macrophages around tumours, tumour vascular tissues, thrombosis, etc.

As universally admitted, liposome solutions are aqueous suspensions of microscopic, spherically shaped, vesicles whose core may hold entrapped aqueous solutions of substances dissolved in the liposome carrier liquid. These vesicles are usually formed of one or more concentrically arranged molecular double layers (lamellae) of amphipatic compounds, i.e. compounds having a lipophobic hydrophilic moiety directed toward the water phase) and a lipophilic hydrophobic moiety holding the layers together. (See for instance "Liposome Methodology", Ed. L.D. Leserman et al, Inserm 136, 2-8 May 1982). Bioactive substances can be encapsulated within the aqueous phase of the core of liposome vesicles and the suspensions can be injected into the body, whereby they can be made to circulate in the blood or the lymph; as said before, release of the encapsulated substances will then result from the opening or rupture or collapse of the liposomal vesicle membrane. The targeted method is particularly suitable for local administration of toxic substances which, if not targeted, could (and would) otherwise cause significant secondary effects to other organs; such drugs include for instance Amphotericin B or NSAID's or drugs whose administration is required over prolonged periods such as Dexamethasone, insulin, vitamin E, etc. The method is also suitable for administration of thrombolytic agents such as urokinase or streptokinase, or antitumoral compounds such as Taxol etc.

Definitions of the terms "microbubbles" and "microballoons" as used herein are given in the above-referenced publications. For instance, in the present disclosure "microbubble" specifically designates air or gas filled microspheres in suspension in a liquid carrier phase which generally result from the introduction therein of air or a gas in divided form, the liquid phase

preferably also containing surfactants or tensides to control the surface properties thereof and the stability of the bubbles. In the microbubbles, the boundary or envelope around the gas core is mostly evanescent and may simply consists of the gas/liquid interface layer which is generally only a few nanometer thick. The term of "microballoon" designates preferably air or gas microspheres with a tangible material boundary or envelope formed of molecules other than that of the liquid of suspension, for instance, a protein or a polymeric or lipidic membrane, this shell being tens or hundreds of nm thick.

More specifically in the present invention, one will consider that the internal volume of the microbubbles is limited by the gas/liquid interface, or in other words, the microbubbles are only bounded by an envelope involving the molecules of the liquid and surfactants loosely bound at the gas to liquid interface or boundary. In the present invention, the surfactants preferably comprise one or more phospholipids at least in part in laminar or lamellar form. The term "lamellar form" indicates that the surfactants are in the form of thin films involving one or more molecular layers ("lamine" form). Converting such film forming phospholipid surfactants into lamellar form can easily be done by liposome methodology, for instance by pressure homogenisation or by sonication under acoustical or ultrasonic frequencies. In this connection, it should be remembered that, as said above, the liposome vesicles membrane itself is made of phospholipids in lamellar form.

Many surfactants or tensides, including lipids, particularly phospholipids, can be laminarized to correspond to this kind of structure. In this invention, one preferably uses the lipids commonly used for making liposomes, for instance saturated phospholipids, natural

or preferably synthetic, as well as other surfactants or glycerides which can be made into layers or films.

Particularly preferred are the phospholipids selected from neutral phospholipids such as hydrogenated
5 phosphatidyl choline (HSPC), dipalmitoyl-, distearoyl- and diarachidoyl phosphatidylcholine (DPPC, DSPC, DAPC); negatively charged phospholipids such as dipalmitoyl and distearoyl phosphatidic acid (DPPA, DSPA), dipalmitoyl and distearoyl phosphatidylserine (DPPS, DSPS), dipalmitoyl
10 and distearoyl phosphatidylglycerol (DPPG, DSPG); reactive phospholipids such as phosphatidyl ethanolamine derivatives coupled to a polyethyleneglycol, a biotinyl, a glutaryl, a caproyl or a succinyl amine.

The microballoons which are useful in this invention
15 are described in EP-A-0 458 745. They have a tangible envelope made of substantive material, e.g. a polymeric membrane with definite mechanical strength. In other terms, they are microspheres of flexible solid material in which the air or gas is more or less tightly confined.
20 Microballoons made by sonication of viscous protein solutions like 5% serum albumin and having diameters in the 1-20 μm range, and stabilised by denaturation of the membrane forming protein may also be used.

The polymer which constitutes the envelope or bounding
25 membrane of the injectable microballoons preferred in this invention can be made from most hydrophilic, biodegradable physiologically compatible polymers. Among such polymers, which may be natural or synthetic, one can cite polysaccharides of low water solubility, polycyano-
30 acrylates, polylactides and polyglycolides and their copolymers, copolymers of lactides and lactones such as γ -caprolactone, δ -valerolactone, polypeptides, and proteins such as gelatin, collagen, globulins and albumins. The great versatility in the selection of synthetic polymers
35 is another advantage of the present invention since, as with allergic patients, one may preferably avoid using

microballoons made of natural proteins (albumin, hemoglobin) like in US-A-4,276,885 or EP-A-0 324 938. Other suitable polymers include poly-(ortho)esters (see for instance US-A-4,093,709; US-A-4,131,648; US-A-4,138,344; US-A-4,180,646); polylactic and polyglycolic acid and their copolymers, for instance DEXON (J. Heller, *Biomaterials* 1 (1980), 51; poly(DL-lactide-co- γ -caprolactone), poly(DL-lactide-co- δ -valerolactone), poly(DL-lactide-co- γ -butyrolactone), polyalkylcyanoacrylates; polyamides, polyhydroxybutyrate; polydioxanone; poly- β -aminoketones (*Polymer* 23 (1982), 1693); polyphosphazenes (*Science* 193 (1976), 1214); and polyanhydrides. References on biodegradable polymers can be found in R. Langer et al., *Macromol. Chem. Phys.* C23 (1983), 61-126. Polyaminoacids such as polyglutamic and polyaspartic acids can also be used as well as their derivatives, i.e. partial esters with lower alcohols or glycols. One useful example of such polymers is poly-(t.butyl-glutamate). Copolymers with other amino acids such as methionine, leucine, valine, proline, glycine, alanine, etc. are also possible. Other derivatives of polyglutamic and polyaspartic acid with controlled biodegradability have been reported (see WO 87/03891; US 4,888,398 and EP-A-0 130 935) all incorporated herein by reference.

25 The gases to fill the microspheres of this invention include air, and most gases common in the field of echogenic gases, for instance SF₆, CF₄, C₂F₆, C₃F₆, C₃F₈, C₄F₆, C₄F₈, C₄F₁₀, C₅F₁₀, C₅F₁₂, air, oxygen, nitrogen, carbon dioxide, noble gases, and mixtures thereof.

30 Innocuous, low boiling liquids which will vaporise at body temperature or by the action of remotely applied energy pulses, like C₆F₁₄, are also usable as a volatile confinable microparticle component in the present invention.

35 The confined gases may be at atmospheric pressure or under pressures higher or lower than atmospheric; for

instance, the confined gases may be at pressures equal to the hydrostatic pressure of the carrier liquid holding the liposomes and the gas filled microspheres.

In the present invention, the gas-filled microspheres
5 may be more or less closely associated with the liposomes, i.e. they may simply be admixed with the liposome vesicles whereby they will statistically distance from each other. Alternatively, the liposome vesicles and the gas-filled microspheres can be organised to have affinity for each
10 other, for instance they may each be provided with the molecular components of a conjugate pair. As an example, an antigen may be incorporated in the liposome membrane and an antibody in the microspheres, or vice-versa, so that antigen-antibody conjugation will cause the
15 microspheres and the liposome vesicles to couple with each other. Other coupling systems involving donors and receptors in the classes of substances listed below are also possible: amphetamines, barbiturates, sulphonamides, monoamine oxydase inhibitor substrates, hormones, enzymes,
20 lipids, ligands specific of cellular membranes, antihypertensive agents, neuro-transmitters, aminoacids, oligopeptides, radio-sensitizers, steroids (e.g. estrogen and estradiol), mono- and polyclonal antibodies as well as fragments thereof, carbohydrates (such as glucose
25 derivatives), fatty acids, muscarine receptors and substrates (such as 3-quinuclidinyle benzilate), dopamine receptors and substrates (such as spiperone), biotin, peptides and proteins capable of binding specific receptors, benzodiazepine receptors and substrates.

30 Systems involving multiple coupling sites are also possible. For instance, in a particular embodiment of the present invention's method and formulation, the envelopes of both liposome vesicles and gas microspheres are provided with biotin coupling sites and a suspension
35 thereof in an aqueous carrier liquid is admixed with avidin, whereby both the liposome vesicles and gas

microspheres will coalesce together by coupling with avidin.

The liposomes used in this invention are preferably of the long-lived (stealth) type, i.e. resistant to capture by the RES. Stealth liposomes are disclosed in documents such as *J. Pharmacy & Pharmacol.* 39 (1987), 52P; EP-A-0 354 855, WO 91/05545; EP-A-0 759 785; EP-A-0 731 690; *Biochimica et Biophysica Acta* 1126 (1992), 255-260, and "Stealth Liposomes" Edited by D. Lasic and F. Martin (1995) CRC Press, London, all publications incorporated herein by reference.

Particularly preferred embodiments of the present invention involve liposomes which comprise three components: A. a neutral lipid, for example, a nonionic or zwitterionic lipid or their derivatives; B. a negatively or positively charged lipid, and C. a lipid bearing a functional component, for example N-biotinyl-PE or PEG-PE. Cholesterol or cholesterol derivatives can be used to replace a part of component A, as generally known to the skilled person.

The lipids used to make the liposomes can be selected from a group comprising: lipids and phospholipids such as soy lecithin, partially refined lecithin, hydrogenated phospholipids, lysophosphate, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cardiolipin, sphingolipids, gangliosides, cerebroside, ceramides, other esters analogue of phosphatidylcholine (PAF, lysoPAF); synthetic phospholipids such as L- α -lecithin (dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine, dilinoleoylphosphatidylcholine, distearoylphosphatidylcholine, diarachidoylphosphatidylcholine); phosphatidylethanolamine derivatives, such as 1,2-diacyl-sn-glycero-3-phosphoethanolamine, 1-acyl-2-acyl-sn-glycero-3-phosphoethanolamine, dinitrophenyl- and dinitrophenylamino caproylphosphatidylethanolamine, 1,2-diacyl-sn-glycero-3-phospho-

ethanolamine-N-polyethylene glycol (PEG-PE), N-biotinyl-PE, N-caproylamine PE, N-dodecylamine-PE, N-MPB-PE, N-PDD-PE, N-succinyl-PE, N-glutaryl-PE; phosphatidyl glycerols such as dipalmitoylphosphatidylglycerol, distearoyl-phosphatidylglycerol; phosphatidic acids (1,2-diacyl-sn-glycero-3-phosphate salt, 1-acyl-2-acyl-sn-glycero-3-phosphate sodium salt; phosphatidylserine such as 1,2-diacyl-sn-glycero-3-[phospho-L-serine] sodium salt, 1-acyl-2-acyl-sn-glycero-3-[phospho-L-serine] sodium salt, lysophosphatidic acid; cationic lipids such as 1,2-diacyl-3-trimethylammoniumpropane (TAP), 1,2-diacyl-3-dimethylammoniumpropane (DAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N',N''-trimethylammonium chloride (DOTMA); polymerizable lipids such as diyne PC, diynePE for example 1,2-bis(10,12-tricosadiynoyl-sn-glycero-3-phosphocoline; phospholipids with multivarious headgroups such as phosphatidylethanol, phosphatidylpropanol and phosphatidylbutanol, phosphatidylethanolamine-N-monomethyl, 1,2-distearoyl(dibromo)-sn-glycero-3-phosphocoline; phospholipids with partially or fully fluorinated fatty acid chains.

Emulsifying or surfactant agent may also be incorporated in the liposomes or used for liposome preparation, such as Pluronic®, Poloxamer®, Span®, Brig®, Tweens®, Triton-X®; fluorinated surfactants such as Zonyl®.

For preparing the liposome suspensions useful in the present invention, one can apply the conventional techniques in the field, for instance that disclosed in the aforesaid documents and the following one: Liposomes as Drug Carriers by G. Gregoriadis, Wiley & Sons, New-York (1988).

For instance, as disclosed in GB-A-2,134,869, microspheres (10 μ m or less) of a hydrosoluble carrier solid (NaCl, sucrose, lactose and other carbohydrates) are coated with a phospholipid mixture; then, by dissolution

of this coated carrier in an aqueous phase, one will obtain liposomic vesicles. In GB-A-2,135,647 insoluble particles, e.g. glass or resin microbeads are coated by moistening in a solution of lipids in an organic solvent followed by removal of the solvent by evaporation. The lipid-coated microbeads are thereafter contacted with an aqueous carrier phase, whereby liposomic vesicles will form in that carrier phase.

It is of particular interest to note that in the present invention, the generation of the microbubbles, the eventual burst of which will help split the liposome vesicles membrane and liberate the encapsulated content, is directly (although partly) related the formation of the liposomes. Indeed, as disclosed in EP-A-0 474 833, admission of air or a gas in a suspension of liposomes will provide stable microbubble suspensions containing from about 10^7 to 10^{10} microbubbles/ml, or more. Also, according to the same document, similar bubble suspensions will result from exposing for a time to air or a gas formulations of dried laminar phospholipids (which can be compared to liposomes stored dry), and thereafter admixing with a carrier liquid. Hence, it is of interest in the present invention (although not compulsory) to start with liposomes suspensions or solutions prepared by any known technique, and thereafter introduce air or a gas, whereby a stable suspension of microbubbles will form stabilised by the presence of the surfactants in lamellar form. Of course, the material making the liposome walls shall have to be modified within the scope of the present invention, i.e., for instance by admixing therewith or covalently grafting thereon foreign molecules designed for coupling as described before. Alternatively, one may also start with "unloaded" liposome vesicles, i.e. vesicles not having yet a bioactive substance encapsulated therein. Then, before or after air or a gas is introduced into the liposome solution to provide a desired suspension of

microbubbles, loading of the liposome vesicles can be effected as disclosed in EP-A-0 514 523.

5 In an embodiment, a dry powder formulation of liposomes containing bioactive media encapsulated therein can be prepared according to document US-A-4,229,360, the liposome wall-forming material containing an agonist coupling precursor (e.g. biotin). Then, the liposome suspension is regenerated using an aqueous carrier liquid containing an antagonist (e.g. avidin), whereby bubbles
10 will form together with the liposome vesicles, both stabilized by the biotin-containing lipids and coupling via the avidin in the solution.

In a variant, the liposome preparations and the gas-filled microsphere formulations can of course be prepared
15 individually and admixed together before administration. They may also be administered individually in which case the administration is effected sequentially in any order with or without delay between the injections i.e. to delay interference of the microbubbles and the liposome
20 vesicles. In certain applications or modes of treatment several injections of microbubbles may be envisaged to assist release of the liposome content at several sites or for repeated release of the liposome active ingredient at the same site.

25 As said before, microballoons with confined air or gas are also usable according to the invention to help opening liposome vesicles. In this case, the microballoons are prepared separately from the liposomes, preferably according to the techniques disclosed in EP-A-0 458 745,
30 and thereafter admixed with the suspension of liposomes of interest. Naturally also, the envelope of the microballoons will preferably contain a coupling precursor designed to eventually conjugate with a receptor of the liposome membrane (or vice-versa). Practical achievements
35 of such an embodiment are disclosed in the experimental part hereafter.

In order to implement the method of the invention, one will administer the preparations according to usual routes, e.g. intravenous, perfusion, etc. for instance, one can inject into the circulation of subjects by usual
5 means (IV or otherwise) targeted (or non-targeted) preparations as described above containing in admixture liposomes with trapped bioactive media and microspheres (microbubbles or microballoons) with confined air or gas therein. After a time, when the injected material has
10 reached a targeted organ or tissue site in the body, energy pulses are applied from the outside (for instance above or on the skin in relation with the site) to cause the gas containing particles to explode; the cavitation energy thus released by the explosion brings about the
15 opening of the liposome envelope and the discharge of the encapsulated materials.

Energy pulses required to explode the gas-filled microspheres are preferably sonic or ultrasonic pulses. In this connection see the publication by M.W. Miller et al.
20 in Ultrasound in Med. & Biol. 22 (1996), 1131-1154. In broad, transducer systems can be applied directly to the body or through a water-path couplant with the frequencies in the range from about 0.3 to 3 MHz. In a preferred embodiment, there is used a modified ultrasound probe for
25 monitoring displacement of the bubbles after administration and the destruction thereof when appropriate at the application site. The collapsing of the bubbles is then depicted by a dramatic change of the reflected echo signal. The monitoring signal is in the
30 range of 1 MHz to 10 MHz and preferably between 2 and 7 MHz.

In view of the various formulation embodiments to be possibly used in the present invention, systems of precursors developed comprise components to be admixed
35 before use and delivered commercially for instance in a

kit form for easier storage and shipping. These precursor systems may include the following embodiments:

5 (A) Solution (or suspensions) of liposomes having bioactive substances encapsulated therein. The solution is then treated with air or a gas, for instance infused before application by means of a syringe or otherwise.

10 (B) Solution (or suspensions) of liposomes having bioactive substances encapsulated therein and a suspension of air- or gas-containing microspheres (stable microbubbles or microballoon) to be admixed therewith.

15 (C) The kit which comprises dried liposomes having bioactive substances encapsulated therein in stabilised powder form and a suspension in a carrier liquid of air- or gas-containing microbubbles or microballoons. Both components are to be admixed before use.

20 (D) The kit which may comprise dried liposomes having bioactive substances encapsulated therein in stabilised powder form, microballoons in dry powder form, or microbubble precursors as pulverulent laminarized phospholipids stored in contact with air or a gas and an
25 administrable carrier liquid, said components to be admixed before use.

(E) In a simplified variant, the kit may comprise dried liposomes stored in stabilised powder form in
30 contact with air or a gas and having bioactive substances encapsulated therein and an administrable carrier liquid, which may be admixed before use, whereby a stable suspension of microbubbles is formed due to the stabilising effect of the phospholipids.

35 As already mentioned, the method of the invention based on microbubble burst acoustic cavitation can be used

not only to promote liposome lysis for drug delivery and contrast enhancement in ultrasound imaging, but also to modify cell permeability for gene transfection or expression. The liposomes may be thermo-sensitive, fusogenic, pH-sensitive, stealth (e.g. PE-PEG) with or without specific homing factors and loaded with different therapeutic, imaging or genetic substances. Preferably, the liposomes are unilamellar, a structure, which enables high drug encapsulation capacity (i.e. high active substance/lipid ratio), and a low shear stability under acoustic cavitation.

The following Examples further illustrate the invention.

15 Example 1

A) Biotin-labeled LUV (large unilamellar vesicles) liposomes.

There were dissolved in 150 ml of a mixture (1:2) of chloroform and methanol at 50°C 0,75 g of hydrogenated soy phosphatidyl choline (HSPC, from Nattermann Chemie, Germany), 50 mg of dipalmitoylphosphatidic acid (DPPA, from Sygena, Switzerland), and 10 mg of N-biotinyl Cap-PE (Avanti Polar Lipids, USA). To this were added 200 g of 1 mm glass beads (Polyscience Inc., USA) and the whole was homogenized in a homogenizer. After removing the solvent on the rotavapor, the residue was suspended in 200 ml of buffer solution (10 mM TRIS + 0,9% NaCl, pH 7.2) containing 10% of optical tracer drug (carboxyfluorescein) and the mixture heated to 60°C to hydrate the lipids. The beads were removed and the liposome solution extruded 5 times through 1 μ m polycarbonate filter membranes; then the solution was dialyzed against the same buffer to eliminate untrapped substances. After dialysis, the solution was checked (Coulter counter), the mean diameter of the liposome vesicles being about 1.3 μ m.

B) Biotin labeled microbubbles

In 150 ml of buffer (10 mM TRIS + 0,9% NaCl, pH 7.2) were dispersed at 65°C 200 mg of dipalmitoylphosphatidyl glycerol (DPPG) and 200 mg of distearoylphosphatidyl-
5 choline (DSPC), all from Sygena, 10 mg of N-biotinyl Cap-PE and 5 g of Pluronic® F-108. After cooling to room temperature, the solution was placed into an emulsifier apparatus equipped with a Polytron® head and emulsified (10,000 rpm) for 2 min under an atmosphere of
10 perfluorobutane (C₄F₁₀) to provide a milky bubble suspension. The upper foam layer was discarded and the solution allowed to settle. The top layer of bubble suspension was collected and resuspended in TRIS-NaCl buffer; thereafter, the decantation operation was repeated
15 twice, whereby the bubbles in the final purified suspension had a mean size of 2.6 µm at a concentration of 5x10⁸ bubbles/ml.

C) Ultrasonic release of carboxyfluorescein (CF) from
20 liposomes

Three 205 µl different samples were prepared as follows:

a) 20 µl of liposome solution (A) + 185 µl of TRIS-
25 NaCl buffer

b) 20 µl of liposome solution (A) + 5 µl of TRIS-NaCl buffer + 180 µl of microbubble solution (B)

c) 20 µl of liposome solution (A) + 5 µl of avidin solution (1 mg/ml in TRIS buffer) + 180 µl of microbubble
30 solution (B).

The samples placed in Eppendorff tubes were subjected for 10 min to the effect of ultrasound in a Branson 5200 apparatus (47 KHz, 0.2 W/cm²). After treatment, the samples were centrifuged and the fluorescence of the tracer
35 released in the supernatant measured with a Kontron SFM-25 fluorimeter (excitation at 480 nm; emission at 520 nm).

Identical samples (untreated) were used as control. The results are gathered in the Table below

Table 1

Sample	CF release (%)	
	No ultrasound (control)	Ultrasound treated
a	2.3	4.1
b	10	23.6
c	9.7	53.4

5 As seen from the foregoing results the maximal delivery of liposome entrapped substance occurs when the bubbles couple with the liposomes via conjugation with avidin.

10 Example 2

MLV liposomes (MLV = multilamellar vesicles) were prepared at the concentration of 10 mg (of mixture of lipids)/ml (of aqueous phase) using a 75:20:5 (w/w) mixture of DSPC/cholesterol/DPPA. The water phase was a 10
15 mM solution of CF in buffer. Hydration of the lipid mixture (liposome vesicles formation) was effected by heating to 65°C under mild agitation for 10 min.

The samples to be tested were made of 100 μ l of liposome suspension plus various quantities of the
20 microbubbles preparation (B) disclosed in Example 1 (see the Table below). Then, for testing, the samples were further diluted to make 6 ml with TRIS buffer and circulated in a thin-wall plastic tubing (f = 4 mm) immersed in a constant 37°C bath with a peristaltic pump.
25 Pulses from a 8550 Tabor generator, amplified with a A-150 ENI RF amplifier, were applied with a 1 MHz focused transducer (Panametric Inc., USA) placed at 9 cm from the tube. The acoustic pressure was measured in the tube with a hydrophone connected to a digital scope (DL-4100 from

Yokogawa, Japan). The following further experimental parameters were applied: Pulse length, 10 μ s; burst number, 100; pressure amplitude in the tube (peak to peak), 1.6 Mpa; exposure time, 3 min; flux rate 15 ml/min.

5 The results are gathered in the next Table

Table 2

Sample	CF release (μ mol)
Liposomes only	1.0
+ 0.1 ml B (from Ex. 1)	1.9
+ 0.5 ml B	7.7
+ 2.5 ml B	15.9

Example 3

10 A suspension of MLV liposomes was prepared as in Example 2. A portion thereof (LUV-1) was converted to LUV by repeated freeze and thaw, followed by five 1 μ m membrane ex-trusions. Another portion (LUV-2) was further extruded through membranes of successively 0.6, 0.4 and
15 0.2 μ m. The samples to be tested were admixed with the microbubble suspension (B) to produce a liposome/microbubble volume ratio of 1:5. The samples were tested for CF release as indicated in Example 1. The results are gathered in the next table.

20

Table 3

Liposome s	Size (nm)	Encapsulat ion ratio (μ l/mg)	CF release (%)
MLV	810	2.3	16
LUV-1	630	8.1	41
LUV-2	260	1.3	10

Example 4

This example illustrates the influence of various parameters such as transducer frequency, output power, flow rate, exposure time, etc. on the gas microbubble-ultrasound induced liposome lysis.

Example 2 was repeated with a constant bubble/liposome concentrations and different ultrasound exposures. The results have shown that the change in transducer frequency from 1 to 2.25 MHz lowers the degree of release of CF from liposomes under the condition where all other parameters were kept constant. Similar observation was made for changes in the flow rate. The higher the flow the lower is the number of the exploded or destroyed microbubbles.

It has been observed that the microbubble destruction was more efficient at higher acoustic powers having as a direct consequence higher release of CF from liposomes. Hence it may be said that the degree of liposome lysis was proportional to the increase in amplitude applied.

The effect of the exposure time was apparently dependent on different settings of power, frequency and flow rate. The liposome lysis was complete when all microbubbles in the suspension were destroyed. However, during a continuous infusion of the microbubbles, the total liposome lysis increased and remained high as long as the microbubble infusion was maintained.

Experimental results (3 min US irradiation, liposomes 500 μ l, bubbles 2.5 ml) :

	Frequency variation at 1.5 MPa, 10 ml/min		Acoustic pressure variation at 1 MHz and 10 ml/min		Flow rate variation at 1 MHz and 1.5 MPa		
	1 MHz	2.25 MHz	0.5 MPa	1.5 MPa	5 ml	10 ml	15 ml
Bubble %*	81.9	68.5	69.5	81.9	94.4	81.9	70.3
Lysis %**	18.7	5.1	7.3	18.7	25.3	18.7	13.2

* % of bubbles destroyed by US irradiation, determined by Coulter.

** Liposome lysis determined by CF release.

These data show that the lysis of liposomes relates closely to the amount of bubbles destroyed by ultrasound (sonolysis).

Example 5

Large unilamellar liposomes (LUV) were prepared according to M.H. Gaber et al., *Int. J. Rad. Oncol. Bio. Phys.* 36 [5] (1996), 1177-1187. A molar ratio mixture (100:50:30:6) of DPPC (dipalmitoylphosphatidyl choline), HSPC, cholesterol, and PE-PEG (distearoylphosphatidyl ethanolamine derivatized with polyethyleneglycol 1900) was dissolved in an organic solvent (see Example 1), and thereafter the obtained solution was allowed to evaporate in contact with a surface so as to form a film of the phospholipids on that surface. Then a 10 mM solution of CF in TRIS (10 mM + 0.9% NaCl, pH 7.4) was added in quantity required to form a 5 mg/ml solution of liposomes; hydration was effected by heating above the transition point and the liposome solution was extruded 5 times through membranes of decreasing pore size. The mean bubble size, measured by light scattering (Nycom apparatus) was about 140 nm.

Samples were prepared by admixing with the microbubble preparation of Example 1, this being also in the same proportion. Table 4 below shows the CF release after exposition of the samples to ultrasonic energy as in Example 1 for 10 min at various temperatures. The data also include controls (no bubbles, no ultrasound) as indicated. They clearly demonstrate the effect of temperature. Note also that in the absence of the "catalyzing" influence of the gas-containing microbodies, the effect of the ultrasound is not much over that of temperature.

Table 4

	CF release (%) at t°C		
Sample	25	37	41
Heat only	3	17	28
Heat + US	4	17	31
Heat + US + bubbles	16	32	54

Another aspect of using the cavitation energy liberated in a medium by the explosion of gas-filled microbodies is to act on the droplets of an emulsion of pharmaceutically acceptable liquids in a carrier phase. One can therefore convey the admixture of emulsion and microbubbles to a selected area in the body and when there, one will trigger the disruption of the droplets by the remote controlled disintegration of the bubbles. The liquid in the droplets can have bioactive substances dissolved therein which will then distribute in the area of interest. In a variant, if sufficiently low boiling, this liquid will simply vaporize and produce a plethora of new bubbles and enhanced echo signal. Many other aspects of using the localized supply of energy from exploding bubbles could be envisaged.

20 Example 6

1 g of dipalmitoyl phosphatidyl glycerol (DPPG, Sygena, Switzerland) and 10 mg of N-Biotinyl Cap-PE (Avanti Polar Lipids, USA) were dissolved in 100 ml of distilled water containing 3 grams of Pluronic®-F108 (a non-ionic surfactant). A clear solution was obtained at 60°C under agitation. This solution was mixed with a gas (ex. C₄F₁₀) in a high speed homogenizer (Polytron®, 10, 000 rpm) for few minutes. An opaque suspension containing between 10⁸ and 10⁹ of gas microbubbles/ml with a size

distribution between 0.7 and 20 μm was obtained. To remove the surfactant, the free (non-incorporated) biotinyl molecules and narrow the microbubble size distribution, the suspensions were repeatedly decanted
5 (washed) several times with water until all surfactant in the suspension was removed (this was controlled by the IR or HPLC). The size distribution and microbubble number may be equally tailored by controlling the duration of decantation and the volume of the supernatant phase
10 recovered (bubble phase). Typically, three decantations were sufficient. In the case where the homing or biomolecules were unstable in aqueous solutions, the microbubble suspension were frozen (e.g. below -18°C) and stored until use.

15 As the surfactants or detergents were used only to facilitate the lipid solubilization and gas microbubble formation, they were removed after the microbubble formation. All surfactants capable of dissolving, co-solubilising or dispersing the phospholipids in aqueous
20 medium can be utilized. Examples of such surfactants are Pluronic[®], Polaxmer[®], Tween[®], Span[®], Chaps (non-denaturing zwitterionic detergent often used for membrane biochemistry) and numerous hydrocarbon surfactants (sodium alkyl sulfate, etc.), fluorocarbon surfactants (e.g.
25 perfluoro alkyl polyoxyethylene), ionic or non-ionic. As the principal element of the microbubble stabilising shell, many phospholipid molecules may be utilized (e.g. phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol, etc.), but for the method in this example the
30 negatively charged phospholipids are preferred because of their co-solubility in water in the presence of other surfactants. Many perfluorocarbon containing synthetic lipids can also be used in this technique for microbubble preparation. Moreover, a mixture of more than two
35 surfactants or of several lipid molecules can be used in

this preparation, which gives often microbubbles with interesting properties and a high yield of microbubbles.

5 This example demonstrates that "surfactant or detergent depletion" method (similar to the process used in liposome preparation) may be employed to incorporate the homing factor into the microbubbles giving them specific properties for targeting in vivo.

Claims

1. A method of delivering a biologically active substance to a selected site in the body of a patient
5 comprising the steps of:

(i) injecting into the circulation of said patient an effective amount of an administrable formulation comprising, as a suspension in a carrier liquid, microspheres carrying an entrapped physiologically
10 acceptable gas and liposome vesicles filled with said biologically active substance;

(ii) allowing said formulation to reach the selected site through the circulation of said patient, and

(iii) ultrasonically irradiating said site so as to
15 cause the microspheres to explode and the entrapped gas to expand in the carrier liquid, the energy of expansion of said gas causing the liposome vesicles to open and release the biologically active substance at said site.

20 2. The method of claim 1, wherein the microspheres are exploded by irradiation with ultrasonic pulses having frequencies of about 0.3 to 3 MHz.

3. The method of claim 2, wherein the ultrasonic
25 pulses are provided by a standard or a modified echography probe adapted to simultaneously monitor the reflected echo signal and thereby provide an image of the irradiated site.

30 4. A formulation for delivery of a biologically active substance to a selected target site in the organism of a patient comprising an aqueous suspension of liposomes filled with said active substance, characterized in that said formulation further comprises an effective amount of
35 a physiologically acceptable gas- or air-filled microspheres.

5. The formulation of claim 4, wherein the microspheres are microbubbles bounded by a liquid/gas interface or microballoons bounded by a tangible membrane.

5

6. The formulation of claim 4, wherein the carrier liquid comprises amphipatic compounds to stabilize the gas-containing microspheres against premature collapse.

10

7. The formulation of claim 6, wherein the amphipatic compounds are phospholipids, preferably saturated phospholipids.

15

8. The formulation of claim 7, wherein stabilization of the microbubbles is brought about by a monolayer of phospholipids at the gas/liquid interface.

20

9. The formulation of claim 5, wherein the membrane of the microballoons is made from a natural or a synthetic polymer.

25

10. The formulation of claim 4, wherein the gas in the microspheres is selected from SF₆, CF₄, C₂F₆, C₃F₆, C₃F₈, C₄F₆, C₄F₈, C₄F₁₀, C₅F₁₀, C₅F₁₂, C₆F₁₄, air, oxygen, nitrogen, carbon dioxide, noble gases, and mixtures thereof.

30

11. The formulation of claim 7, wherein the phospholipids are selected from neutral phospholipids such as hydrogenated phosphatidyl choline (HSPC), dipalmitoyl-, distearoyl- and diarachidoyl phosphatidylcholine (DPPC, DSPC, DAPC); negatively charged phospholipids such as dipalmitoyl and distearoyl phosphatidic acid (DPPA, DSPA), dipalmitoyl and distearoyl phosphatidylserine (DPPS, DSPS), dipalmitoyl and distearoyl phosphatidylglycerol (DPPG, DSPG); reactive phospholipids such as phosphatidyl

35

ethanolamine derivatives coupled to a polyethylenglycol, a biotinyl, a glutaryl, a caproyl or a succinyl amine.

12. The formulation of claim 1, wherein the liposome
5 vesicles and the air or gas- filled microspheres are optionally organized to have affinity for each other.

13. The formulation of claim 12, wherein the liposome
10 vesicles and the microspheres are each provided with the respective components of a conjugate pair.

14. The formulation of claim 13, wherein an antigen is
present in the liposome membrane and an antibody in the
microspheres, or vice-versa, so that antigen-antibody
15 conjugation will cause the liposome vesicles and the
microspheres to be brought together.

15. The formulation of claim 12, wherein both the
liposome vesicles and the microspheres are provided with a
20 donor coupler element and the formulation further
comprises a multisite acceptor element, whereby donor and
acceptor will become conjugated and the liposome vesicles
and the microbodies are brought together.

25 16. A kit comprising precursor components in sterile
separate form which upon admixture provide a formulation
according to anyone of claims 4-15.

17. A kit according to claim 16, comprising:
30 a) an aqueous suspension of liposomes having bioactive
substances encapsulated therein;
b) a sterile physiologically acceptable gas or air;
and
c) means to introduce said gas into said solution, so
35 as to form a suspension of microbubbles in said liposome
solution.

18. A kit according to claim 16, comprising:
a) a suspension of liposomes having bioactive substances encapsulated therein;

5 b) a suspension of physiologically acceptable gas- or air-containing microspheres in a carrier liquid.

19. A kit according to claim 16, comprising:

10 a) a powder of dried liposomes having bioactive substances encapsulated therein in stabilized form;

b) a suspension of physiologically acceptable gas- or air-containing microspheres in a carrier liquid.

20. A kit according to claim 16 comprising:

15 a) dried liposomes having bioactive substances encapsulated therein in stabilized powder form;

(b) a gas-filled microballoons in dry powder form, or microbubble precursors as pulverulent laminarized saturated phospholipids stored in contact with air or a
20 physiologically acceptable gas and, optionally, hydrosoluble stabilisers; and

(c) an administrable aqueous carrier liquid.

21. A kit according to claim 16 comprising:

25 a) dried liposomes stored in stabilized powder form having bioactive substances encapsulated therein and in contact with sterile air or a physiologically acceptable gas; and

(b) an administrable aqueous carrier liquid.

30

22. The kit according to any one of claims 16-21, wherein the liposomes are made from natural or synthetic lipids and/or phospholipids selected from hydrogenated phosphatidyl choline (HSPC), lysophosphate, phosphatidic
35 acid, phosphatidyl glycerol, phosphatidylcholine,

phosphatidylserine, phosphatylethanolamine, cholesterol and their derivatives counterparts.

23. The kit according to any one of claims 16-22, in which the gas in the microspheres is selected from SF₆, CF₄, C₂F₆, C₃F₆, C₃F₈, C₄F₆, C₄F₈, C₄F₁₀, C₅F₁₀, C₅F₁₂, C₆F₁₄, air, oxygen, nitrogen, carbon dioxide, noble gases, and mixtures thereof.

24. The formulation according to anyone of claims 4-15 for use in therapeutic or gene treatment and/or imaging of organs or tissue in patients.

25. The kit according to anyone of claims 16-23 for use in therapeutic or gene treatment and/or imaging of organs or tissue in patients.

26. A method of making of the formulation according to anyone of claims 4-15 using the kit of claims 16-23.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K41/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 28873 A (UNGER EVAN C ;FRITZ THOMAS A (US); MATSUNAGA TERRY (US); RAMASWAMI) 22 December 1994 cited in the application see claims 1-25 ---	1
X	WO 95 07072 A (SCHERING AG ;WEITSCHIES WERNER (DE); HELDMANN DIETER (DE); HAUFF P) 16 March 1995	1-26
Y	see claims 1,6,8,10,17; figures ---	1-26
X	WO 94 28874 A (UNGER EVAN C ;FRITZ THOMAS A (US); MATSUNAGA TERRY (US); RAMASWAMI) 22 December 1994	1
Y	see page 35, line 33 - page 36, line 6; claims 1-5,47 -----	1-26



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

*** Special categories of cited documents :****"A"** document defining the general state of the art which is not considered to be of particular relevance**"E"** earlier document but published on or after the international filing date**"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)**"O"** document referring to an oral disclosure, use, exhibition or other means**"P"** document published prior to the international filing date but later than the priority date claimed**"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention**"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone**"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.**"&"** document member of the same patent family

Date of the actual completion of the international search

1 June 1999

Date of mailing of the international search report

09/06/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Berte, M

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9428873 A	22-12-1994	US 5580575 A	03-12-1996
		AU 696056 B	27-08-1998
		AU 6953794 A	03-01-1995
		AU 684088 B	04-12-1997
		AU 7094894 A	03-01-1995
		AU 8840698 A	04-02-1999
		CA 2164843 A	22-12-1994
		CA 2164846 A	22-12-1994
		CN 1125393 A	26-06-1996
		CN 1125394 A	26-06-1996
		EP 0802788 A	29-10-1997
		EP 0707471 A	24-04-1996
		JP 9501410 T	10-02-1997
		JP 8511523 T	03-12-1996
		WO 9428874 A	22-12-1994
		US 5542935 A	06-08-1996
		US 5773024 A	30-06-1998
		US 5733572 A	31-03-1998
		US 5705187 A	06-01-1998
		US 5770222 A	23-06-1998
		US 5656211 A	12-08-1997
WO 9507072 A	16-03-1995	DE 4330958 A	16-03-1995
		DE 4416818 A	16-11-1995
		AU 7655194 A	27-03-1995
		AU 7729998 A	10-09-1998
		CA 2171303 A	16-03-1995
		EP 0717617 A	26-06-1996
		HU 74509 A	28-01-1997
		JP 9502191 T	04-03-1997
		NO 960973 A	08-03-1996
WO 9428874 A	22-12-1994	US 5580575 A	03-12-1996
		US 5585112 A	17-12-1996
		US 5542935 A	06-08-1996
		AU 696056 B	27-08-1998
		AU 6953794 A	03-01-1995
		AU 684088 B	04-12-1997
		AU 7094894 A	03-01-1995
		AU 8840698 A	04-02-1999
		CA 2164843 A	22-12-1994
		CA 2164846 A	22-12-1994
		CN 1125393 A	26-06-1996
		CN 1125394 A	26-06-1996
		EP 0802788 A	29-10-1997
		EP 0707471 A	24-04-1996
		JP 9501410 T	10-02-1997
		JP 8511523 T	03-12-1996
		WO 9428873 A	22-12-1994
		US 5773024 A	30-06-1998
		US 5733572 A	31-03-1998
		US 5705187 A	06-01-1998
		US 5770222 A	23-06-1998
		US 5656211 A	12-08-1997
		AU 1004399 A	04-03-1999
		AU 2185095 A	19-06-1995
		AU 683900 B	27-11-1997
		AU 7043194 A	03-01-1995

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9428874 A		CA 2177713 A	08-06-1995
		CN 1137748 A	11-12-1996
		EP 0712293 A	22-05-1996
		EP 0740528 A	06-11-1996
		JP 8511526 T	03-12-1996
		JP 9506098 T	17-06-1997
		WO 9428780 A	22-12-1994
		WO 9515118 A	08-06-1996
		US 5853752 A	29-12-1998
		US 5776429 A	07-07-1998
		CN 1125389 A	26-06-1996

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.